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Stimulation of Symbiotic N_2 Fixation in *Trifolium repens* L. under Elevated Atmospheric pCO_2 in a Grassland Ecosystem[1]

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Symbiotic N_2 fixation is one of the main processes that introduces N into terrestrial ecosystems. As such, it may be crucial for the sequestration of the extra C available in a world of continuously increasing atmospheric CO_2 partial pressure (pCO_2). The effect of elevated pCO_2 (60 Pa) on symbiotic N_2 fixation (^{15}N-isotope dilution method) was investigated using Free-Air-CO_2-Enrichment technology over a period of 3 years. *Trifolium repens* was cultivated either alone or together with *Lolium perenne* (a nonfixing reference crop) in mixed swards. Two different N fertilization levels and defoliation frequencies were applied. The total N yield increased consistently and the percentage of plant N derived from symbiotic N_2 fixation increased significantly in *T. repens* under elevated pCO_2. All additionally assimilated N was derived from symbiotic N_2 fixation, not from the soil. In the mixtures exposed to elevated pCO_2, an increased amount of symbiotically fixed N (+7.8, 8.2, and 6.2 g m^{-2} a^{-1} in 1993, 1994, and 1995, respectively) was introduced into the system. Increased N_2 fixation is a competitive advantage for *T. repens* in mixed swards with pasture grasses and may be a crucial factor in maintaining the C:N ratio in the ecosystem as a whole.

In recent years significant attention has been paid to the possible impact of increasing atmospheric pCO_2, one of the major factors in the global climate change phenomenon, on ecosystems. Elevated pCO_2 is likely to affect C cycling by stimulating photosynthesis and, therefore, the primary productivity of terrestrial ecosystems, which may result in an increase in the sequestration of C into the biosphere and organic matter. However, primary productivity of an ecosystem may be limited by other environmental factors such as irradiation, temperature, or availability of water and mineral nutrients (Bazzaz, 1990; Gifford, 1992). N availability is one of the key factors limiting crop yield (e.g. Kirkby, 1981). If greater CO_2 availability results in increased plant growth, then elevated pCO_2 will ultimately lead to increased N demand at the single-plant level (Ingestad, 1982). Therefore, the extent of the CO_2 response at the plant level could be limited by N availability. If so, legumes, which can fix atmospheric N_2 in symbiosis, would have an advantage over other plants.

Symbiotic N_2 fixation is considered to be the major process causing the introduction of N into most terrestrial ecosystems. It can be assumed that the sequestration of C and N into an ecosystem occur in concert (Granhall, 1981; Gifford, 1992; Hartwig et al., 1996), so symbiotic N_2 fixation may not be regulated only by the N demand of the individual plant, but also indirectly by the N demand of the ecosystem as a whole (Hartwig et al., 1996). Numerous studies covering a wide range of N_2-fixing legumes and woody species have demonstrated an increase in total nitrogenase activity per plant under elevated pCO_2 (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982; Murphy, 1986; Norby, 1987; Arnone and Gordon, 1990). However, instantaneous measurements of nitrogenase activity provide no information about the relative contribution of N assimilation from symbiosis versus N from soil or fertilizer. Moreover, these measurements do not provide an estimate of the amount of N introduced by symbiotic N_2 fixation into an ecosystem. In a world of increasing atmospheric pCO_2, such information is needed to predict how individual plants and the ecosystem as a whole will respond to an alteration in the C:N balance.

Integrated measurements of N_2 fixation using the ^{15}N-isotope dilution method will ultimately lead to a better understanding of the processes regulating C sequestration into ecosystems. The results of various CO_2-enrichment experiments have shown that the stimulation of the above-ground biomass production is stronger in legumes than in nonlegumes (Newton et al., 1994; Lüscher et al., 1996). However, the below-ground response of *Trifolium repens* is relatively weak compared with that of *Lolium perenne* (Jongen et al., 1995). All of these facts are consistent with the possibility that symbiotic N_2 fixation plays a key role in the response of grassland ecosystems to elevated pCO_2.

The aim of these experiments was to examine symbiotic N_2 fixation of field-grown *T. repens* under long-term elevated CO_2 conditions using FACE technology. This technology allows investigations of the CO_2 response of large field plots without artifacts related to microclimatic conditions such as temperature, irradiation, and wind. The results indicate that

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Abbreviations: FACE, Free Air Carbon-dioxide Enrichment; %Nsym, percentage of plant N derived from symbiotic N_2 fixation; pCO_2, partial pressure of CO_2.

there is an increase in symbiotic N_2 fixation in response to the CO_2-induced increase in the N demand in the individual plant and in the ecosystem as a whole.

MATERIALS AND METHODS

FACE Experiment and Plant Material

FACE technology (Hendrey et al., 1992; Lewin et al., 1994) was used to investigate the long-term effects of elevated pCO_2 on a model grassland ecosystem in the field. The study was carried out at 550 m above sea level in Eschikon, which is near Zurich, Switzerland. Each of the three blocks consisted of a fumigated (60 Pa CO_2) and a control (ambient pCO_2) ring (18 m diameter) situated at least 100 m apart. Within each block, the plots had been subjected to the same crop rotation before the establishment of the experiment. The CO_2 fumigation was begun May 31, 1993, and continued until the end of the growing season of that year. In 1994 and 1995, however, the fumigation lasted for the entire growing season during the daytime. There was a 1-min average of 60 Pa $\pm$10% within 92% of the fumigated time ($\pm$20% within 99% of the time) for the three rings.

The soil at the experimental site was a fertile, eutric cambisol (clay loam according to the U.S. classification) with a potassium and phosphorus content adequate for intensively managed grassland. In the early spring of each experimental year the plots of the first two blocks were fertilized with P_2O_5 (12 g m^{-2}), K_2O (29 g m^{-2}), and Mg (1.6 g m^{-2}). In the third block the amount of fertilizer was increased by 35% to counterbalance differences in nutrient availability.

In mid-August 1992, *Trifolium repens* cv Milkanova was sown either as a monoculture (0.8 g m^{-2}) or as a mixture (0.4 g m^{-2}) with *Lolium perenne* cv Bastion (1.6 g m^{-2}). Each plot measured 2.8 $\times$ 1.9 m. Two different N treatments and two different harvesting regimes were used. In 1993, the swards were harvested either five (frequent defoliation) or three (infrequent defoliation) times during the fumigation period. Before the start of CO_2 fumigation (May 31, 1993) the swards were harvested in mid-April and in mid-May. These cuts were not considered for the analysis of the 1993 data. In 1994 and 1995, defoliation frequency was increased to eight and four harvests, respectively. Cutting height was approximately 5 cm above ground level.

Each year in the fall the plots were treated with vincolzolin (DuPont) (0.1 g m^{-2}) to eradicate *Sclerotinia trifoliorum*. In the spring, benomyl (Siegfried, Zofingen, Switzerland) (5 g m^{-2}) was applied to provide protection against *Fusarium oxysporium*.

N and ^{15}N Application

N was applied at two different rates: either 10 or 42 g m^{-2} a^{-1} (equivalent to 100 or 420 kg ha^{-1} a^{-1}) in 1993, and either 14 or 56 g m^{-2} a^{-1} (140 or 560 kg ha^{-1} a^{-1}) in 1994 and 1995. The amount of N for one regrowth period was adjusted to the biomass production. This was expected to be highest in the spring, and progressively lower toward the end of the growing season. In the frequently defoliated treatment the annual dosage in 1993 was split 25, 20, 17, 15, 15, and 8% for the first through the sixth regrowth periods, respectively. In 1994 and 1995 the corresponding values were 16, 15, 12.5, 12.5, 12.5, 12.5, 10, and 9% for the eight regrowth periods. The infrequently defoliated treatment received 30, 25, 25, and 20% of the annually applied N in all 3 years. Applications were made on the day after harvesting and for the first regrowth period at the beginning of April (1994 and 1995) or in mid-April (1993).

N was supplied as a solution of NH_4NO_3 (1 L m^{-2}). All plots were watered with 1 L m^{-2} of water following the N application. The harvest area (0.9 $\times$ 1.1 m) was fertilized with ^{15}N-enriched NH_4NO_3 (ammonium and nitrate were equally labeled) (Isotec, Miamisburg, OH, and Matheson, Secaucus, NJ). In the first two experimental seasons the ^{15}N atom% excess was 0.4% for the high-N treatment and 1.6% for the low-N treatment. In the third year the ^{15}N atom% excess was decreased to 0.3 and 1.3% for the high- and low-N treatments, respectively. The remainder of each plot (4.33 m^2) was supplied with an equal amount of unlabeled NH_4NO_3 (Fluka Chemie, Buchs, Switzerland).

Sampling

Plant material from the central part (0.45 $\times$ 0.55 m) of the ^{15}N-labeled area (0.9 $\times$ 1.1 m) was separated into *T. repens*, *L. perenne*, and unsown species. *T. repens* and *L. perenne* were dried at 65°C for 48 h.

After the last harvest in 1993 and 1994, the remaining above-ground (below cutting height) and below-ground biomass was harvested, with three above-ground samples (100 cm^2) and two soil cores (5 cm diameter, 50 cm depth) being taken in all of the *T. repens* monocultures. Before drying, the root material was washed using Gillison's hydropneumatic elutriation system (Smucker et al., 1982).

All dried plant material was chopped into small pieces (Fuchs-Mühle, type M.M.125 h., Fuchs, Vienna, Austria) and then ground (sequentially by a Cyclotec 1093 sample mill, Tecator, Höganäs, Sweden, and by a ball mill, type MM2, Retsch, Arlesheim, Switzerland) to a very fine powder. After redrying (35°C for 24 h), the samples (1 mg) were weighed in tin caps (0.04 mL, Lüdi, Flawil, Switzerland). The samples were analyzed for ^{15}N and N concentration by a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) in the laboratory of Dr. C. van Kessel (University of Saskatchewan, Saskatoon, Canada). Leaf material (50 mg) from two harvests (May and July 1994) prepared in the same way was analyzed for N concentration on an elemental analyzer (LECO CHN-1000, LECO Corp., St. Joseph, MI).

Calculation of %Nsym

%Nsym is a yield-independent parameter and was calculated for each regrowth period according to McAuliffe et al. (1958):

$$\%\text{Nsym} = \left(1 - \frac{^{15}\text{N atom\% } - \text{excess in fixing crop in monoculture or mixture}}{^{15}\text{N atom\% } - \text{excess in intercropped nonfixing reference crop}}\right) \times 100$$

L. perenne grown in mixtures served as reference crop. *L. perenne* has similar N uptake and rooting patterns as *T. repens* (Boller and Nösberger, 1988) and is therefore a suitable reference plant for studies on N_2 fixation by *T. repens*.

Elevated pCO_2 may induce changes in rooting depth. Thus, a homogeneously labeled soil profile is a prerequisite for an accurate application of the ^{15}N-dilution method (Danso et al., 1993), which was achieved by applying ^{15}N in solution to the same area at the beginning of each regrowth period. This procedure is considered to minimize errors due to mismatch between the reference and the N_2-fixing crop (Danso et al., 1993). To further check the suitability of the selected reference plant, plant material from unsown, nonfixing plants was collected in the same area and analyzed as described above. Only small differences were observed in ^{15}N atom% excess between the nonfixing plant species and these were most likely due to differences in N uptake and rooting patterns (Witty, 1983). However, these differences were not influenced by the CO_2 treatments, indicating that possible CO_2-induced changes in rooting depth were insignificant for the N-uptake pattern. In November 1994, 4 weeks after the last fertilization, the ^{15}N atom% excess of the plant-available N in different soil horizons (0–15, 15–30, or 30–45 cm) was examined in separate plots with high-N *L. perenne* monocultures that had been treated exactly as the *T. repens* monocultures. Mineral N was extracted from 100 g of sieved soil with 1 M KCl solution (200 mL) according to the procedure described by Keeney and Nelson (1982), and analyzed for ^{15}N atom% excess. The results showed that the ^{15}N-label was evenly distributed throughout the profile (0.166, 0.164, and 0.159 ^{15}N atom% excess for the upper, the middle, and the lowest soil layer, respectively). This ensures that any possible CO_2-induced change in the rooting depth of the plants would not influence the accuracy of the determination of N_2 fixation in *T. repens*.

Statistical Analysis

The experimental design was a split-split-plot design. pCO_2 was the main-plot factor and defoliation frequency was the subplot factor. Because the blocks and interactions between three or four experimental factors were statistically insignificant, they were pooled into the specific error terms. Weed growth was particularly severe in 1995, so yields were corrected proportionally when weed dry matter was higher than 5% of the total yield. Analysis of variance was carried out using a statistical analysis package (SAS, SAS Institute, Cary, NC). LSD values are indicated for comparison between the two main-plot treatment means (averaged over all subplot treatments) and for comparison between the two main-plot treatment means at the same or different subplot treatments (i.e. means of any two treatment combinations) (Gomez and Gomez, 1984).

RESULTS

Effect of Elevated pCO_2 on N Yield and N Concentration of *T. repens* as Influenced by N Supply, Defoliation Treatment, and Sward Type

In general, elevated pCO_2 increased the annual above-ground N yield of frequently defoliated *T. repens* swards (Table I). When averaged across all treatments, the N yield under elevated pCO_2 increased by 24.0, 17.5, and 6.3% in 1993, 1994, and 1995, respectively, but only the 1993 increase was statistically significant. The N-yield response to CO_2 in *T. repens* was not affected by N supply or sward type. The infrequently defoliated swards responded similarly to elevated pCO_2 (data not shown), resulting in an insignificant interaction between CO_2 treatment and defoliation frequency. In all 3 years the effect of CO_2 on the N yield of *T. repens* was more pronounced in the mixed swards (42, 32, and 31% in 1993, 1994, and 1995, respectively) than in the monocultures, where the initially strong CO_2 effect (16% in 1993) declined to 10 and −4% in 1994 and 1995, respectively (Table I). However, there was no significant interaction between CO_2 and sward type. Although the total N yield of *T. repens* increased under elevated pCO_2 in 1993 and 1994, the N concentration in the above-ground plant material produced under elevated pCO_2 was in general significantly reduced (Table II). Regardless of which CO_2 treatment was applied, N supply and sward type always significantly affected the N yield of *T. repens* (with the exception of the 1995 crop) (Table I).

%Nsym

For all 3 years the annually averaged %Nsym, a yield-independent parameter, was higher in *T. repens* grown under elevated pCO_2 than in that grown under ambient pCO_2 (Fig. 1). This effect of CO_2 on %Nsym was observed for both N treatments and for both sward types throughout all three growing seasons. In 1993 the annually averaged %Nsym increased from 55.0% under ambient pCO_2 to 67.0% under elevated pCO_2, whereas the response of %Nsym to high pCO_2 was lower in 1994 (46.4 versus 52.0%) and 1995 (44.0 versus 49.4%) than in 1993. Except in 1993, when elevated pCO_2 evoked a more pronounced increase in %Nsym in the monocultures (from 42.7 to 55%) than in the mixtures (from 67.3 to 75.4%), no interactions were found between CO_2 treatment and sward type or N supply. Out of 84 data pairs, %Nsym was higher under elevated pCO_2 in a total of 80 cases (Fig. 1). In the infrequently defoliated swards %Nsym responded similarly to the CO_2 enrichment (data not shown).

High N supply decreased the %Nsym significantly in all 3 years (Fig. 1). The %Nsym for *T. repens* grown in mixtures was significantly higher than for *T. repens* grown in monocultures in all 3 years (Fig. 1).

Effect of Elevated pCO_2 on N Yield from Symbiosis versus N from the Soil in *T. repens*

Averaged across all treatments, the amount of N derived from symbiosis under elevated pCO_2 was increased by 57, 38, and 23% in 1993, 1994, and 1995, respectively (Table I); however, this was statistically significant only in 1993. In the monocultures, there was a tendency for above-ground N yield originating from the soil or fertilizer to decrease under elevated pCO_2 (Table I). Thus, any additional N incorporated under elevated pCO_2 was derived from the

Table I. *The contribution of symbiotically derived N (Nsym) and soil (Nsoil) to total annual above-ground N yield (Ntot) of T. repens under ambient and elevated pCO_2 in frequently defoliated monocultures and mixtures at two N supplies over three growing seasons*

Means and $\text{LSD}_{0.05}$ of three replicates are shown.

Year	Parameter		Monoculture			Mixture		CO_2 Means
			Low N[a]	High N[b]		Low N[a]	High N[b]	
					$g\ m^{-2}\ a^{-1}$			
1993	Ntot	35 Pa	40.6	41.0		25.3	12.3	29.8
		60 Pa	49.8	44.9		35.2	18.2	37.0
		LSD			10.2[c]			3.86[d]
	Nsym	35 Pa	21.4	14.8		19.6	7.8	15.9
		60 Pa	35.1	21.6		29.2	13.7	24.9
		LSD			8.5[c]			4.3[d]
	Nsoil	35 Pa	19.2	26.2		5.7	4.5	13.9
		60 Pa	14.7	23.3		6.0	4.5	12.1
		LSD			4.2[c]			0.5[d]
1994	Ntot	35 Pa	50.4	50.8		33.1	20.7	38.8
		60 Pa	55.6	55.9		40.4	30.5	45.6
		LSD			16.2[c]			14.4[d]
	Nsym	35 Pa	27.4	13.8		20.6	7.0	17.2
		60 Pa	31.6	19.5		29.2	14.7	23.7
		LSD			9.3[c]			10.9[d]
	Nsoil	35 Pa	23.0	37.0		12.5	13.7	21.6
		60 Pa	24.0	36.4		11.2	15.8	21.8
		LSD			8.5[c]			11.0[d]
1995	Ntot	35 Pa	50.6	52.0		25.3	23.1	37.8
		60 Pa	48.8	50.0		32.6	30.6	40.2
		LSD			22.6[c]			22.0[d]
	Nsym	35 Pa	27.4	15.5		17.1	7.3	16.8
		60 Pa	30.0	16.5		24.0	12.7	20.7
		LSD			9.5[c]			14.0[d]
	Nsoil	35 Pa	23.2	36.5		8.2	15.8	20.9
		60 Pa	18.8	33.5		8.6	17.9	19.7
		LSD			11.2[c]			11.3[d]

[a] Low N, 10 $g\ m^{-2}\ a^{-1}$ in 1993; 14 $g^{-m}\ a^{-1}$ in 1994 and 1995. [b] High N, 42 $g^{-2}\ a^{-1}$ in 1993; 56 $g^{-m}\ a^{-1}$ in 1994 and 1995. [c] Comparison between two main-plot treatment (tr) means at the same or different subplot tr (i.e. means of any treatment combinations) (Gomez and Gomez, 1984). [d] Comparison between two main-plot tr means averaged over all subplot tr (Gomez and Gomez, 1984).

symbiosis (Table I). This effect was less evident in the intercropped clover.

N yield of roots and stolons harvested at the end of the growing season in 1993 and 1994 (early November) showed a similar pattern for the contribution of N derived from symbiosis versus N from the soil, as was seen for the above-ground plant material (Table III). Total N yield of stolon and root fractions tended to increase under elevated pCO_2. The major contributor to this was symbiotic N_2 fixation (Table III).

DISCUSSION

Effect of Elevated pCO_2 on N Yield of *T. repens* and on the N Concentration in the Above-Ground Biomass

Under elevated pCO_2, the total above-ground N yield of *T. repens* tended to increase, but this CO_2 effect was statistically significant only in 1993 (Table I). This is partly attributed to the fact that the factor CO_2 was weakly tested because its error term has only four degrees of freedom. The CO_2-induced increase in N yield was more pronounced and persistent in the intercropped *T. repens* than in the monocropped type. The difference in CO_2 response between the mixtures and the monocultures can be attributed at least in part to the increased proportion of total yield represented by *T. repens* under elevated pCO_2 (from 30% under ambient pCO_2 to 42% under elevated pCO_2 averaged over 3 years). The response pattern of *T. repens* to elevated pCO_2 is independent of the defoliation frequency, illustrated by the insignificant interaction between CO_2 and defoliation frequency (data not shown).

The N concentration in the above-ground plant material was reduced under elevated pCO_2 (Table II). This commonly documented CO_2 effect (Conroy and Hocking, 1993) is attributed to C-assimilated accumulation in leaves and/or an improvement in N efficiency under elevated pCO_2 (Andrews and Lorimer, 1987; Badger, 1992; Morell et al., 1992). The reduction in N concentration appears to be less pronounced in legumes than in other grassland species (Larigauderie et al., 1988; Luo et al., 1994). Carbohydrate accumulation in plant organs and/or shifts in the composition of the harvested material in favor of plant fractions of low N concentration may contribute to a reduction in the N concentration in the total above-ground biomass. In fact, a consistent decrease in the leaf-to-petiole ratio, known to correspond to a reduced N concentration in the above-

Table II. *Averaged N concentration in above-ground plant material of T. repens under ambient and elevated pCO_2 in frequently defoliated mixed and monocropped swards at two nitrogen supplies during three growing seasons*

Means and $LSD_{0.05}$ of three replicates are shown.

Year		Monoculture			Mixture		CO_2 Means
		Low N[a]	High N[b]		Low N[a]	High N[b]	
				mg g^{-1} dry weight			
1993	35 Pa	46.7	43.9		44.1	43.7	44.6
	60 Pa	45.6	41.6		41.8	39.4	42.1
	LSD			2.30[c]			3.75[d]
1994	35 Pa	47.1	48.5		46.3	47.1	47.3
	60 Pa	41.7	42.6		42.8	42.1	42.3
	LSD			2.81[c]			2.79[d]
1995	35 Pa	45.2	48.3		45.4	50.2	47.3
	60 Pa	43.2	45.0		41.5	48.9	44.7
	LSD			4.89[c]			1.65[d]

[a,b] See Table I. [c,d] See Table I.

ground material in *T. repens* (Soussana and Arregui, 1995), was also observed under elevated pCO_2 in our experiment. Consistent with this observation are the results of specific harvests (May 17 and July 12, 1994) that showed that under elevated pCO_2 the N concentration of leaf blade material was reduced by only 7% compared with the total above-ground material, which was reduced by 10%.

Why Does Elevated pCO_2 Stimulate Symbiotic N_2 Fixation?

Under elevated pCO_2 one might expect that the increased N yield in the above-ground plant material would be the result of a concerted increase in both N from the symbiosis and N from the soil and/or fertilizer. Such a response has been observed in a growth-chamber experiment in which plants were grown in sand (Zanetti et al., 1994); however, in the present field experiment we consistently observed an increased %Nsym under elevated pCO_2 throughout the entire growing season (Fig. 1).

Soil N availability affects the performance of N_2 fixation in legumes. A low supply of N from fertilizer, as well as the presence of associated nonsymbiotic plants competing for N, decrease the soil N availability, which is positively correlated with %Nsym in *T. repens* (Boller and Nösberger, 1987; Nesheim and Oyen, 1994; Seresinhe et al., 1994) and in many other legume species (Hardarson et al., 1991; Nesheim and Oyen, 1994). These observations are clearly confirmed by the present study (Fig. 1). Therefore, the increased %Nsym under elevated pCO_2 must be the result of reduced soil-N availability, which was evoked by a CO_2-induced increased N demand in the system. Therefore, the increased competitive ability of *T. repens* under elevated pCO_2 (Newton et al., 1994; Lüscher et al., 1996) is attributed to its ability to fix N_2 to compensate for the reduced availability of mineral N.

Apart from the N supply and the presence of nonfixing-associated plant species, other processes such as leaching, mineralization, denitrification, and N immobilization influ-

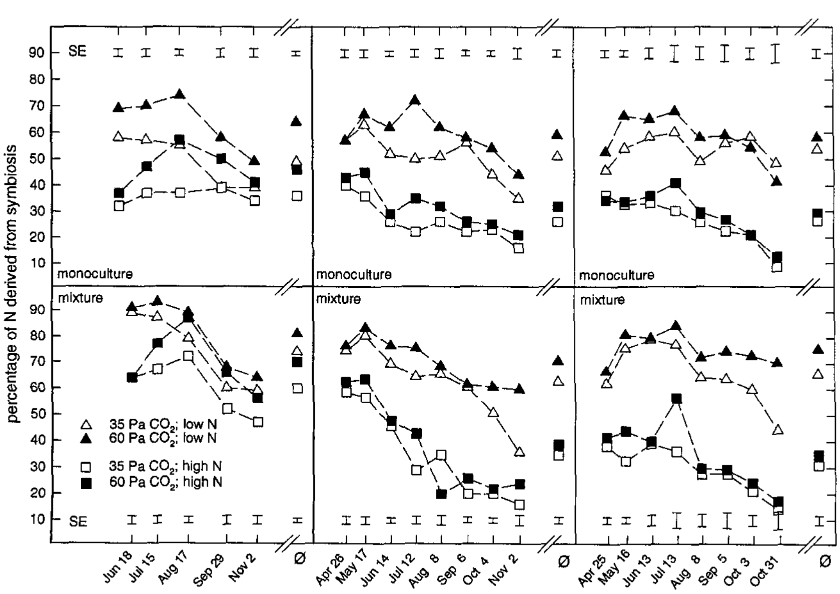

Figure 1. %Nsym as determined from the above-ground plant material under ambient and elevated pCO_2 of frequently defoliated (six cuts a^{-1} in 1993; eight cuts a^{-1} in 1994 and 1995) monocropped and mixed swards over three growing seasons. Low N: 10 g m^{-2} a^{-1} in 1993 and 14 g m^{-2} a^{-1} in 1994 and 1995. High N: 42 g m^{-2} a^{-1} in 1993 and 56 g m^{-2} a^{-1} in 1994 and 1995. The annual average (Ø) is shown at the right side of each graph. Means and SE values of three replicates are shown.

Table III. *The contribution of symbiotically derived N (Nsym) and soil (Nsoil) to the N yield (Ntot) of T. repens roots and stolons under ambient and elevated pCO_2 in frequently defoliated monocultures at the end of the growing seasons 1993 and 1994*

Means and $LSD_{0.05}$ of three replicates are shown.

Year	Parameter		Roots				Stolons			
			Low N[a]		High N[b]	CO_2 Mean	Low N[a]		High N[b]	CO_2 Mean
			$g\ m^{-2}\ a^{-1}$							
1993	Ntot	35 Pa	1.20		1.22	1.21	6.04		5.22	5.63
		60 Pa	1.41		1.82	1.62	7.17		5.59	6.38
		LSD		0.47[c]		1.37[d]		1.61[c]		2.15[d]
	Nsym	35 Pa	0.65		0.46	0.56	3.22		1.92	2.57
		60 Pa	1.10		0.87	0.97	4.96		2.69	3.82
		LSD		0.19[c]		0.68[d]		0.55[c]		1.37[d]
	Nsoil	35 Pa	0.55		0.76	0.66	2.82		3.30	6.12
		60 Pa	0.31		0.95	0.63	2.21		2.90	2.56
		LSD		0.31[c]		0.55[d]		0.72[c]		0.94[d]
1994	Ntot	35 Pa	1.43		1.72	1.58	4.80		5.63	5.21
		60 Pa	2.49		2.43	2.46	6.30		5.98	6.14
		LSD		0.84[c]		1.05[d]		1.69[c]		2.77[d]
	Nsym	35 Pa	0.64		0.46	0.55	2.45		1.44	1.94
		60 Pa	1.59		0.86	1.21	3.97		2.10	3.04
		LSD		0.24[c]		0.45[d]		1.50[c]		1.5[d]
	Nsoil	35 Pa	0.79		1.26	1.03	2.35		4.19	3.27
		60 Pa	0.90		1.57	1.24	2.33		3.88	3.11
		LSD		0.69[c]		0.62[d]		3.00[c]		1.50[d]

[a,b] See Table I. [c,d] See Table I.

ence the amount of mineral N available to *T. repens* and, therefore, affect %Nsym. All of these processes may be affected by pCO_2. Under elevated pCO_2, increases in rhizodeposition, as well as in the total quantity of C:N ratio of litter and root material (van Veen et al., 1991; Jongen et al., 1995), may ultimately alter below-ground processes involved in nutrient cycles (Curtis et al., 1994; Norby, 1994). Along with the suggested increase of N immobilization into the expanded microbial biomass (Diaz et al., 1993), enhanced denitrification may also reduce N availability. Higher soil moisture resulting from the lowered water use of plants exposed to elevated pCO_2 (Morison, 1985; Goudriaan and Unsworth, 1990), along with elevated oxygen consumption by the increased microbial activity and root biomass (Jongen et al., 1995), may lower the oxygen partial pressure in the soil and, therefore, favor denitrification activity. Preliminary denitrification measurements suggested higher gaseous N losses from soil under elevated pCO_2 (P. Ineson, unpublished data of the same experiment). Additional support for a decrease in below-ground N availability comes from the observation that nitrate leaching from soil is reduced during winter from a CO_2-fumigated perennial ryegrass plot (Soussana et al., 1996).

Assimilation of soil N was either unchanged or decreased under elevated pCO_2 (Tables I and III), even though Jongen et al. (1995) reported up to a 48% increase in clover root biomass in the same experiment. This observation is consistent with an unchanged or reduced soil-N availability under elevated pCO_2. Moreover, there was little difference in the amount of N derived from the soil, despite the fact that the yield of *T. repens* as a proportion of total forage yield was greater under elevated pCO_2 (Tables I and III). Furthermore, the increase in the total amount of N fixed accounted for all additionally yielded N and, thus, fully compensated for the apparently reduced N availability in the soil under elevated pCO_2 (Tables I and III).

It is known from short-term studies in growth chambers, greenhouses, and open-top chambers that nitrogenase activity is enhanced under elevated pCO_2 in grain legumes (Hardy and Havelka, 1976; Phillips et al., 1976; Masterson and Sherwood, 1978; Finn and Brun, 1982; Williams et al., 1982), N_2-fixing trees (Norby, 1987; Arnone and Gordon, 1990), and forage legumes (Masterson and Sherwood, 1978; Murphy, 1986). Hardy and Havelka (1976) suggested that the pCO_2-induced increase in nitrogenase activity results from an enhanced C availability for the high-energy-demanding N_2 reduction in the nodules. However, there is little evidence in the literature for an increase in specific nitrogenase activity (activity per unit of nodule weight) under elevated pCO_2 (Finn and Brun, 1982; Murphy, 1986; Norby 1987), and recent studies showed that symbiotic N_2 fixation is not directly regulated by the availability of C assimilates either in the whole plant (Hartwig et al., 1990, 1994; Denison et al., 1992) or in the nodules (Weisbach et al., 1996). Nodule oxygen permeability (Hartwig and Nösberger, 1994) and plant N sink strength (Heim et al., 1993; Oti-Boateng and Silsbury, 1993; Parsons et al., 1993; Hartwig et al., 1994; Oti-Boateng et al., 1994) are likely to be the direct regulators of nitrogenase activity. Increased plant biomass production results in an increased N-sink strength (Ingestad, 1982) and, therefore, in an increased N_2 fixation. The increase in total nitrogenase activity under elevated pCO_2 reported by Finn and Brun (1982), Murphy (1986), and Norby (1987) resulted from an increase in nodule weight and number and was not apparent until several days after exposure to elevated pCO_2. Therefore, this increase results from a general CO_2-induced growth response, leading to a higher N demand and, thus, a higher nitrogenase activity per plant, which is a result of an increased nodulation and may lead to a higher N assimilation caused by the symbiosis, as shown in the present experiment. A 2-fold increase in the number of free-living *Rhizobium leguminosarum* bv *trifolii* cells in the rhizosphere of *T. repens* exposed to elevated pCO_2 (Schortemeyer et al., 1996) suggests that the roots were successfully and extensively nodulated.

Symbiotic N_2 Fixation Can Correct the C:N Imbalance in the Ecosystem Caused by Elevated pCO_2

It is known that the C:N ratio in *T. repens* is lower than in nonfixing species. This difference is especially pronounced under elevated pCO_2 (Larigauderie et al., 1988; Luo et al., 1994; Zanetti et al., 1995). As a result, *T. repens* litter decomposes more rapidly than litter from grasses (Gorissen et al., 1995). Thus, recycling of N would be expected to be quicker in a pasture ecosystem in which *T. repens* is the dominant species.

Under elevated pCO_2 a shift from a predominantly C-limited to a predominantly N-limited ecosystem occurs. Therefore, we expected that the mixtures would exhibit a stronger CO_2-induced increase in %Nsym due to a more pronounced reduction in soil N caused by the associated N-demanding *L. perenne*. One reason this did not occur is that the increased N_2 fixation under elevated pCO_2 improved the competitive ability of *T. repens*, resulting in a significantly increased legume yield proportion (from 30% under ambient pCO_2 to 42% under elevated pCO_2 averaged over 3 years). Thus, total demand and uptake of mineral N per area decreased. Consequently, the mineral N concentration in the soil decreased less and the stimulation of N_2 fixation was less pronounced. In summary, the CO_2-induced increased sink for symbiotically fixed N in the mixtures was satisfied not only by an enhanced N_2 fixation in each clover plant, but also by an increased clover yield proportion.

Our results are consistent with the hypothesis that the amount of N_2 that is fixed, which is inversely related to the N content of an ecosystem (Granhall, 1981), increases under elevated pCO_2 as a result of the increased C:N ratio in the ecosystem (Gifford, 1992; Hartwig et al., 1996; Soussana and Hartwig, 1996). Indeed, under elevated pCO_2 the mixtures introduced more symbiotically fixed N (+7.8, 8.2, and 6.2 g m^{-2} a^{-1} in 1993, 1994, and 1995) into the system compared with the control.

The possibility of assimilating more N (increased total N_2 fixation per plant) under elevated pCO_2 enhances the competitive advantage of legumes over nonlegumes. Therefore, over several years, N_2 fixation and N from mineral fertilizers will introduce increased amounts of N into the ecosystem. The previously postulated increase in the C:N ratio will return to a new balanced value. Thus, under elevated pCO_2 legumes may enable additional C sequestration into the plant biomass, and, subsequently, into the ecosystem as a whole.

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